A modified calcium retention capacity assay clarifies the roles of extra- and intracellular calcium pools in mitochondrial permeability transition pore opening

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Calcium homeostasis is essential for cell survival and is precisely controlled by several cellular actors such as the sarco/endoplasmic reticulum and mitochondria. Upon stress induction, Ca^{2+} released from sarco/endoplasmic reticulum stores and from extracellular Ca^{2+} pools accumulates in the cytosol and in the mitochondria. This induces Ca^{2+} overload and ultimately the opening of the mitochondrial permeability transition pore (mPTP), promoting cell death. Currently, it is unclear whether intracellular Ca^{2+} stores are sufficient to promote the mPTP opening. Ca^{2+} retention capacity (CRC) corresponds to the maximal Ca^{2+} uptake by the mitochondria before mPTP opening. In this study, using permeabilized cardiomyocytes isolated from adult mice, we modified the standard CRC assay by specifically inducing reticular Ca^{2+} release to investigate the respective contributions of reticular Ca^{2+} and extracellular Ca^{2+} to mPTP opening in normoxic conditions or after anoxia–reoxygenation. Our experiments revealed that Ca^{2+} released from the sarco/endoplasmic reticulum is not sufficient to trigger mPTP opening and corresponds to ~50% of the total Ca^{2+} levels required to open the mPTP. We also studied mPTP opening after anoxia–reoxygenation in the presence or absence of extracellular Ca^{2+}. In both conditions, Ca^{2+} leakage from internal stores could not trigger mPTP opening by itself but significantly decreased the CRC. Our findings highlight how a modified CRC assay enables the investigation of the role of reticular and extracellular Ca^{2+} pools in the regulation of the mPTP. We propose that this method may be useful for screening molecules of interest implicated in mPTP regulation.

Ca^{2+} homeostasis supports the cell ability to integrate stimuli, trigger Ca^{2+} signals, and ultimately control molecular pathways essential for cellular physiology. This cascade of events is accurately controlled in space and time (reviewed in Ref. 1). Ca^{2+} channeling between the sarco/endoplasmic reticulum and the mitochondria occurs in microdomains known as mitochondria-associated membranes, which have been reported to control specific cell functions (reviewed in Ref. 2) such as mitochondrial bioenergetics, lipid metabolism, and cell fate. Disruption of mitochondria-associated membranes has been associated to different pathologies including hypoxia–reoxygenation (3–5). Ischemia or hypoxia induces a drop in ATP content, which in turn decreases ATP-dependent Ca^{2+} pumps in both sarcolemma (plasma membrane Ca^{2+} ATPase) and sarco/endoplasmic reticulum (SERCA),[3] leading to an increase in cytosolic (6) and consequently mitochondrial (7) Ca^{2+} concentration. This Ca^{2+} overload and enhancing factors like reactive oxygen species (8), partial mitochondrial membrane depolarization (9), pH restoration at reperfusion (10, 11), gangliosides (12), P_i (13), and outer mitochondrial membrane components (14) induce mitochondrial swelling and the opening of mPTP and ultimately promote cell death (1, 15, 16).

Although mitochondrial Ca^{2+} overload has been well-identified as being a main contributor to the latter phenomena, the origin of this Ca^{2+} is still unclear. Little is known about whether the internal Ca^{2+} stores alone are sufficient to promote mPTP opening in cardiomyocytes or whether extracellular Ca^{2+} entry is also required. Growing evidences have involved sarcolemmal channels and transporters such as the connexin and pannexin hemichannels (17) or the Na^+/Ca^{2+} exchanger NCX (18). Their inhibition reduced Ca^{2+} overload and decreased myocardial injury, suggesting the involvement of extracellular Ca^{2+}. However, none of these studies have evaluated the respective participation of this extracellular Ca^{2+} and the internal Ca^{2+} stores to the mitochondria Ca^{2+} overload process. Answering this question may help rationalizing therapeutic strategies relying on Ca^{2+} fluxes modulation.

This maximum ability of Ca^{2+} uptake by mitochondria before mPTP opening and mitochondrial swelling is defined as Ca^{2+} retention capacity (CRC) (reviewed in Ref. 19). This feature of mitochondria may vary according to the cell type, as well as the physiological (*e.g.* oxidative stress and pH) or pathologi-

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[3] The abbreviations used are: SERCA, sarco/endoplasmic Ca^{2+} ATPase; CRC, Ca^{2+} retention capacity; AR, anoxia–reoxygenation; mPTP, mitochondria permeability transition pore; CsA, cyclosporine A; CypD, cyclophilin D; CICR, Ca^{2+}-induced Ca^{2+} release; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; A.U., arbitrary unit(s); ANOVA, analysis of variance.

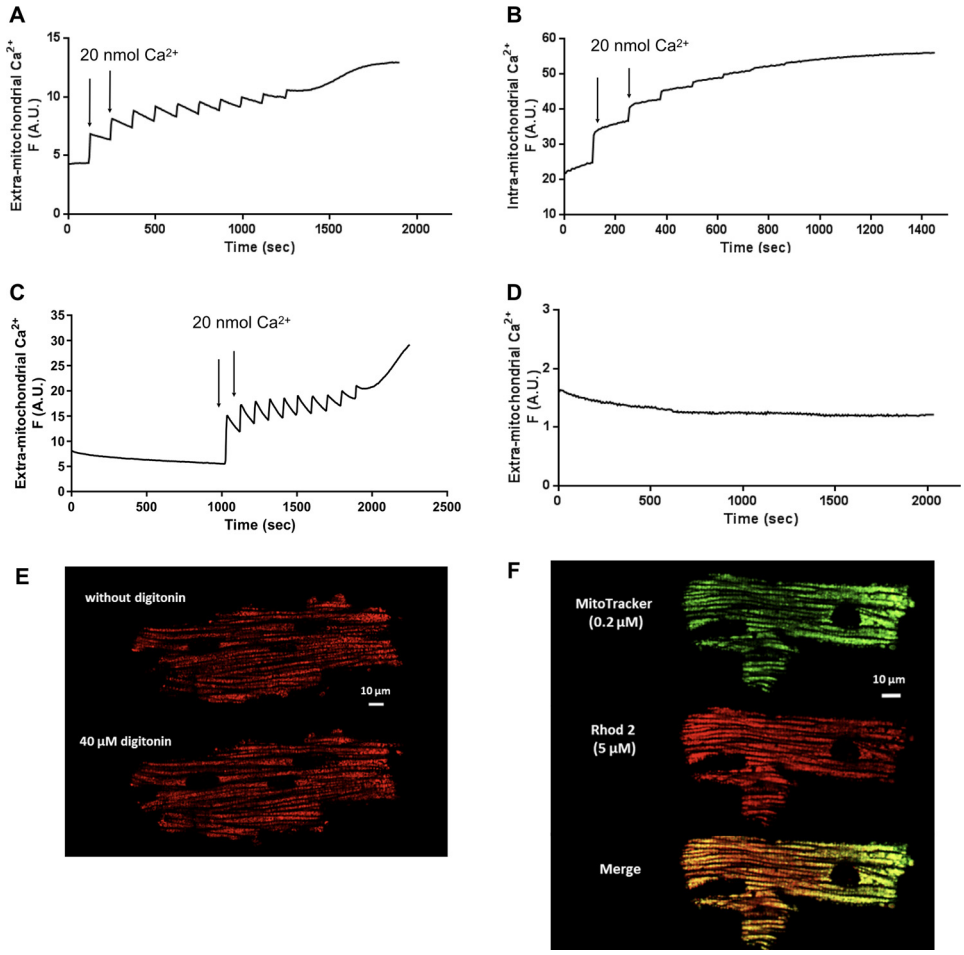


Figure 1. CRC in adult cardiomyocytes. *A* and *B*, CRC was performed in the presence of Ca^{2+} Green-5N probe to detect extramitochondrial Ca^{2+} (*A*) or in the presence of Rhod2-AM probe to detect intramitochondrial free Ca^{2+} (*B*). *C*, cardiomyocytes were incubated for 900 s in CRC medium before CRC measurement. *D*, cardiomyocytes were incubated in CRC medium and recorded for 2000 s in the presence only of Ca^{2+} Green-5N to assess cellular integrity and stability. For all experiments, fluorescence (*F*) is expressed in A.U. Ca^{2+} was added every 120 s by increments of 20 nmol/injection. *E*, confocal microscopy images were acquired after 30 min incubation of cardiomyocytes with 5 μM Rhod2-AM in the presence or absence of 40 μM digitonin. *F*, confocal microscopy images were acquired after 30 min of incubation of cardiomyocytes with 5 μM Rhod2-AM and 0.2 μM MitoTracker Green. 1024 × 1024 pixels images were acquired with an average of four scanning lines. Displayed graphs and images are representative of three independent experiments.

cal cellular state. CRC measurement is experimentally performed on isolated mitochondrial preparations or on cellular models and is conventionally realized with the addition of exogenous Ca^{2+} enabling the simple quantification of the Ca^{2+} amount required to open mPTP. Therefore, the role of reticular Ca^{2+} stores (the major Ca^{2+} store besides the mitochondria) in the mitochondrial Ca^{2+} overload was not studied.

Although several factors are implicated in mPTP opening, numerous studies have shown evidence of the crucial implication of Ca^{2+} overload in mPTP opening especially during ischemia reperfusion (20, 21). In this work we focused particularly on Ca^{2+} contribution. However, we ensure that the other factors contributing to mPTP opening are still present in our experimental condition by using a cellular environment. We investigated concomitantly the role of reticular and extracellular Ca^{2+} in the regulation of mPTP on a model of isolated cardiomyocytes. We modified the standard CRC method by combining the effect of extracellular Ca^{2+} (depicted by the addition of Ca^{2+} pulses) and reticular Ca^{2+} mobilization by stimulating ryanodine receptors using caffeine or ryanodine. We validated our experimental model by showing that, as expected, both pharmacological treatment of cardiomyocytes with cyclosporine A (CsA), an inhibitor of cyclophilin D (CypD), or the genetic ablation of CypD in cyclophilin D knockout (CypD-KO) cardiomyocytes required a greater amount of Ca^{2+} in the mitochondria to open mPTP. In addition, we demonstrated that in both normoxia and anoxia–reoxygenation (AR) conditions, reticular Ca^{2+} stores alone were not sufficient to trigger mPTP opening and that extracellular Ca^{2+} import (Ca^{2+} pulses addition) was required to open mPTP. Finally, we proposed an estimation of these Ca^{2+} sources contributions to mPTP opening.

Results

Validation of the experimental model

As shown in Fig. 1 (*A* and *B*), Ca^{2+} transfers were properly recorded with both probes in isolated cardiomyocyte preparation. Ca^{2+} Green-5N probe reported Ca^{2+} uptake by the mitochondria after each Ca^{2+} pulse, followed by a spontaneous cytoplasmic release indicating mPTP opening (Fig. 1*A*). Conversely, Ca^{2+} uptake by mitochondria was reported by the increase in Rhod2-AM fluorescence after each Ca^{2+} pulse (Fig.

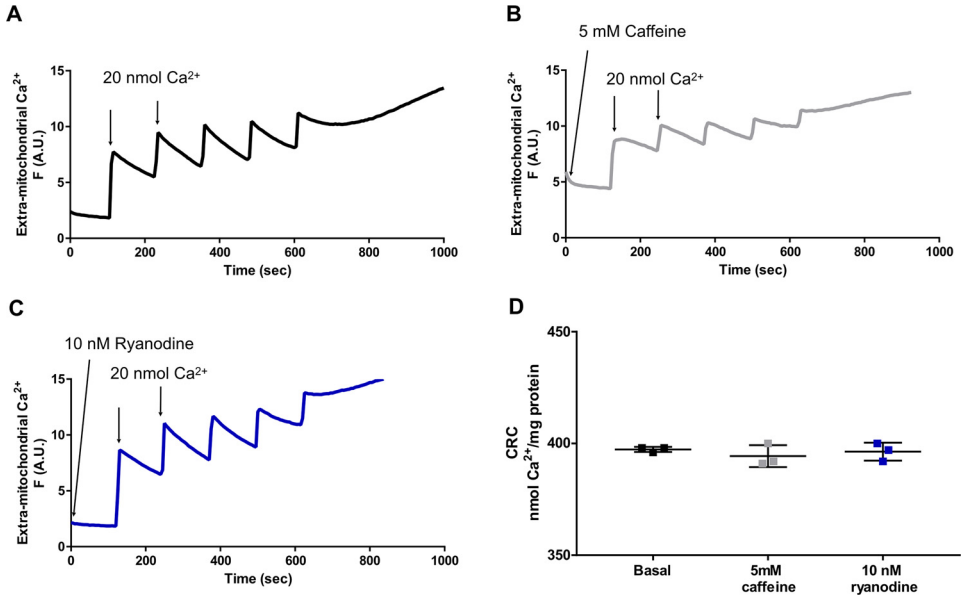


Figure 2. Effect of caffeine and ryanodine on isolated mitochondria. *A–C*, the effect of caffeine and ryanodine was tested on isolated mitochondria. CRC was performed on isolated cardiac mitochondria using Ca^{2+} Green-5N probe in basal condition (*A*) or in the presence of 5 mM caffeine (*B*) or 10 nM ryanodine (*C*). *D*, CRC values were quantified for all conditions. Fluorescence (*F*) is expressed in A.U. Ca^{2+} was added every 120 s by increments of 20 nmol/injection. Displayed graphs are representative of three independent experiments. The CRC values in *D* are presented as means ± S.D. nmol Ca^{2+}/mg protein of three independent experiments (Kruskal–Wallis; $H = 0.6382$, $p = 0.7786$).

1*B*), proving that exogenous Ca^{2+} added during the assay was indeed taken up by the mitochondria. When cardiomyocytes were subjected to ~900 s of incubation before Ca^{2+} pulses (Fig. 1*C*), CRC was still similar to basal condition (Fig. 1*A*) (*i.e.* 10 Ca^{2+} pulses were needed to open mPTP). In addition, cardiac mitochondria *in situ* and permeabilized cellular integrity was preserved during at least 2000 s of incubation in the presence of Ca^{2+} Green-5N probe (Fig. 1*D*). Fluorescence was stable throughout the experiment reflecting the absence of intracellular Ca^{2+} leak and the absence of fluorescence bleaching. We also used Rhod2-AM staining to confirm that the mitochondrial network was not affected in permeabilized cardiomyocytes (40 μM digitonin) (Fig. 1*E*) and that Rhod2-AM colocalized with MitoTracker Green, proving a strict mitochondrial localization of Rhod2-AM staining in our experimental conditions (Fig. 1*F*).

Another control experiment for our method validation was performed on isolated mitochondria (250 μg of protein). Standard CRC was performed in basal conditions (Fig. 2*A*), and in the presence of 5 mM caffeine (Fig. 2*B*) or 10 nM ryanodine (Fig. 2*C*). CRC values were similar in all experiments (Fig. 2*D*), indicating that, at the used concentrations, these ryanodine receptors agonists had no direct mitochondrial targets.

Effects of the reticular Ca^{2+} release on mPTP opening

To study the effect of reticular Ca^{2+} on mPTP opening, CRC was evaluated either by adding exogenous Ca^{2+} pulses alone (Fig. 3*A*, *black panel*) or by using 5 mM caffeine or 10 nM ryanodine followed by exogenous Ca^{2+} pulse addition (Fig. 3*A*, *gray* and *blue panels*, respectively). In basal conditions (Fig. 3, *B* and *C*, *black plots*), 10 pulses (20 nmol Ca^{2+}/pulse) of exogenous Ca^{2+} were needed to open mPTP. The addition of caffeine (Fig. 3*B*, *gray plot*) or ryanodine (Fig. 3*C*, *blue plot*) did not induce fluorescence elevation, suggesting that mitochondria take up the amount of Ca^{2+} released by caffeine or ryanodine stimulation (Fig. 3, *B* and *C*, *insets*). This was confirmed when cardiomyocytes preincubated with 1 μM FCCP were stimulated with 5 mM caffeine: an increase in extramitochondrial fluorescence was observed (Fig. 3*D*), representing the caffeine-induced reticular Ca^{2+} release that cannot be taken up by the mitochondria. The same observation was made after stimulation with 5 mM caffeine in the presence of 1 μM RU360, a specific mitochondrial Ca^{2+} intake inhibitor (Fig. 3*E*).

No massive Ca^{2+} depletion from mitochondria was observed after a 900-s caffeine treatment, indicating that the amount of Ca^{2+} released from reticulum stores was not sufficient to open mPTP. Five additional pulses of 20 nmol of Ca^{2+} were required to induce mPTP opening (Fig. 3, *B* and *C*). The same experiments performed in Rhod2-AM–loaded cardiomyocytes showed an increase in fluorescence after stimulation with 5 mM caffeine (Fig. 3*F*, *gray plot* and *inset*) or 10 nM ryanodine (Fig. 3*F*, *blue plot* and *inset*). Overall, these experiments show that the caffeine-induced Ca^{2+} is quickly and efficiently taken up by mitochondria with a limited Ca^{2+} leak toward cytosol.

Maximal release of reticular Ca^{2+} was triggered by three repetitive stimulations with 5 mM caffeine. However, the amount of Ca^{2+} released was still insufficient, and additional pulses of 20 nmol Ca^{2+} were necessary to open mPTP (data not shown).

After stimulation with 5 mM caffeine or 10 nM ryanodine, the amount of Ca^{2+} necessary to open mPTP significantly decreased to 127 ± 13 nmol/mg protein ($p = 0.0286$) and 137 ± 16 nmol/mg protein ($p = 0.0375$), respectively, when compared with basal condition value (267 ± 22 nmol/mg protein) (Fig. 3*G*). In our conditions, reticular Ca^{2+} released after stimulation with 5 mM caffeine or 10 nM ryanodine represents ~50% (48 ± 7% and 52 ± 6%, respectively) of the Ca^{2+} amount necessary for

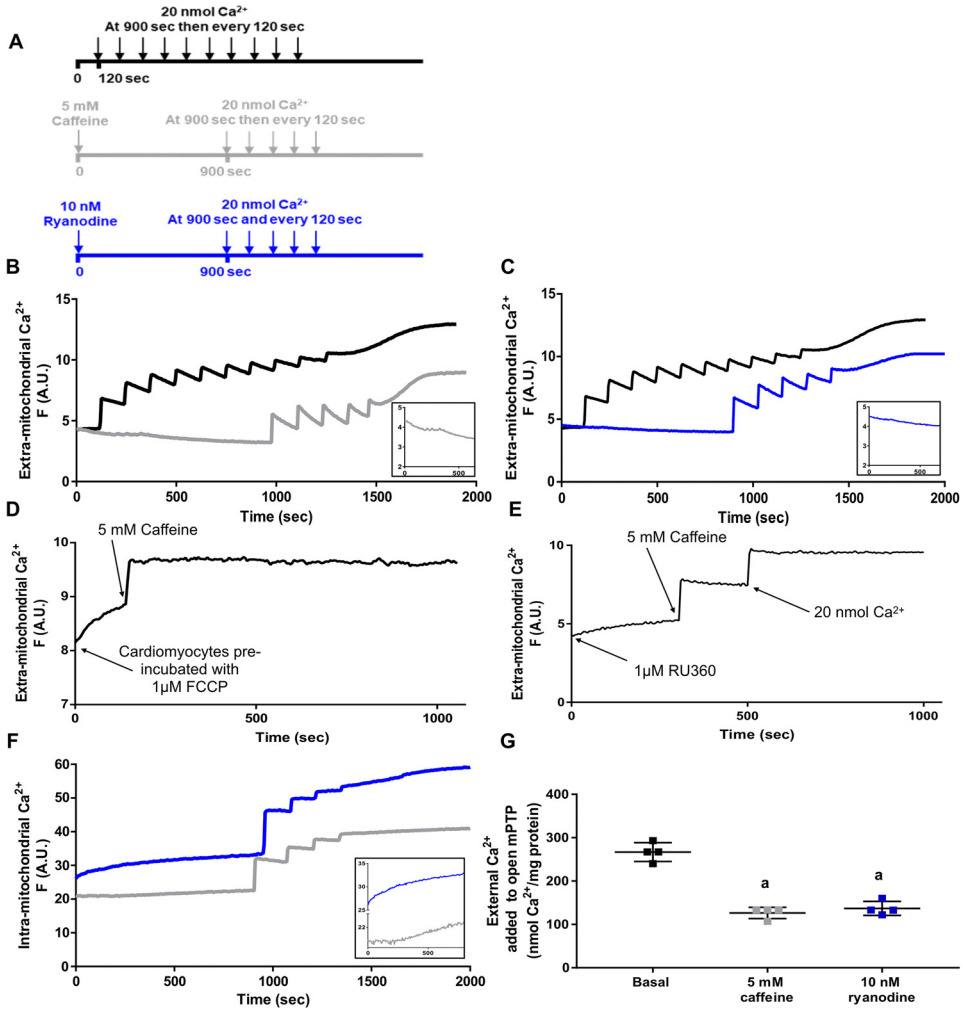


Figure 3. Reticular Ca^{2+} contribution to mPTP opening. *A*, CRC measurement was performed using Ca^{2+} Green-5N probe and conditions. *B* and *C*, Ca^{2+} pulses added in basal condition (*black plots*) were compared with Ca^{2+} pulses added after stimulation with 5 mM caffeine (*B*, *gray plot*) or 10 nM ryanodine (*C*, *blue plot*) in adult cardiomyocytes. *D* and *E*, as a control, extramitochondrial Ca^{2+} fluorescence was measured in cardiomyocytes preincubated with 1 μM FCCP (*D*) or in the presence of 1 μM RU360 (*E*) after reticular Ca^{2+} release stimulation with 5 mM caffeine. Mitochondrial free Ca^{2+} was also stained using Rhod2-AM after 5 mM caffeine or 10 nM ryanodine stimulation (*F*, *gray plot* and *blue plot*, respectively). At 900 s, a series of 20 nmol of Ca^{2+} pulses were necessary to induce mPTP opening. In *B* and *C*, *insets* represent Ca^{2+} Green fluorescence variation from 0 to 500 s and reflect extramitochondrial Ca^{2+} after ryanodine receptor stimulation. In *F*, the *inset* represents Rhod2-AM fluorescence variation from 0 to 500 s and reflects mitochondrial free Ca^{2+} uptake after ryanodine receptor stimulation. *G*, Ca^{2+} amount added to open mPTP in basal condition or after reticular Ca^{2+} stimulation was quantified and presented as dot plots. Fluorescence (*F*) is expressed in A.U. Ca^{2+} was added every 120 s by increments of 20 nmol/injection. The displayed graphs are representative of three or four independent experiments. The values in *G* are presented as means ± S.D. nmol Ca^{2+}/mg protein of four independent experiments (Kruskal–Wallis; $H = 7.69$, $p = 0.0081$), followed by Dunn's post-test. *a*, $p < 0.05$ *versus* basal.

mPTP opening in the basal condition (Fig. 3*G*). This suggests that the reticular Ca^{2+} stores of cardiomyocytes cannot induce mPTP opening by itself.

Involvement of extracellular Ca^{2+} in mPTP opening

To confirm our previous results, we assessed whether preloading cardiac mitochondria with 50% of the Ca^{2+} amount necessary to open mPTP could allow the reticular Ca^{2+} content to drive mPTP opening (Fig. 4*A*). In three independent experiments, the addition of 5 mM caffeine (Fig. 4*B*) or 10 nM ryanodine (Fig. 4*C*) induced a strong and sustained increase in fluorescence intensity indicating a massive mitochondrial Ca^{2+} leak. The reticular Ca^{2+} release induced by either caffeine or ryanodine was sufficient to trigger mPTP opening when cardiomyocytes were loaded with 50% of the Ca^{2+} amount necessary to open mPTP. Both caffeine and ryanodine induced a massive reticular Ca^{2+} release after mitochondria Ca^{2+} preloading, suggesting an absence of or a limited induction of the Ca^{2+}-induced Ca^{2+}-release (CICR) mechanism. This was confirmed by the addition of one 20-nmol Ca^{2+} pulse after the massive fluorescence increase induced by caffeine and ryanodine. This Ca^{2+} pulse was not taken up by the mitochondria, ensuring that the mPTP was indeed opened. As a control of the potential effect of the experiment duration on mPTP opening, we preloaded cardiomyocytes with the same Ca^{2+} amount and recorded fluorescence without the addition of caffeine or ryanodine. In this condition, mPTP did not open, even after 2000 s of incubation time (Fig. 4*D*), demonstrating that mPTP opening observed in Fig. 4 (*B* and *C*) was triggered by caffeine- or ryanodine-induced Ca^{2+} release. In the presence of 110 ± 10-nmol Ca^{2+} load, no additional external Ca^{2+} was needed to open mPTP after internal Ca^{2+} stimulation with caffeine or

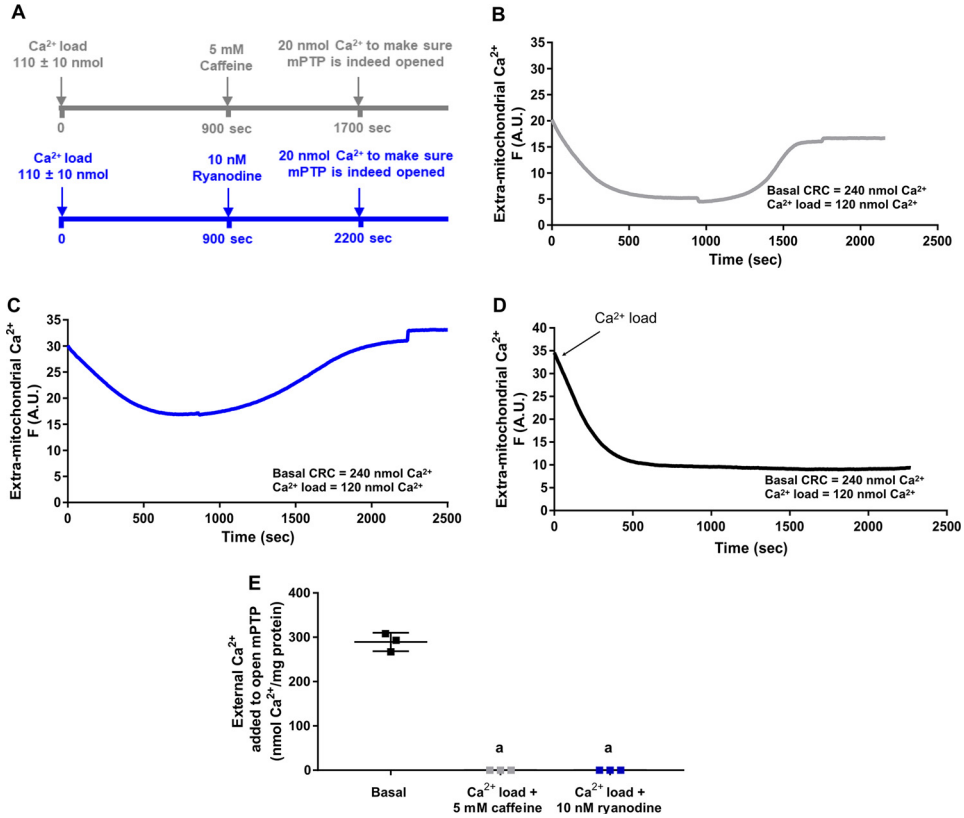


Figure 4. mPTP opening triggered by a combination of external and reticular calcium. *A*, measurements conditions. CRC was measured after the addition of a Ca^{2+} load with 50% of the Ca^{2+} amount necessary to open mPTP. *B* and *C*, at 900 s, ryanodine receptors were stimulated with 5 mM caffeine (*B*) or 10 nM ryanodine (*C*). In both situations, mPTP was opened. An additional pulse of 20 nmol of Ca^{2+} was added but was not taken up by the mitochondria, confirming mPTP opening. *D*, as a control, cardiomyocytes were recorded during 2000 s after the addition of Ca^{2+} load without caffeine or ryanodine stimulation. *E*, the amount of external Ca^{2+} added to open mPTP was quantified in basal condition and in the presence of Ca^{2+} load (110 ± 10 nmol) followed by caffeine or ryanodine stimulation. All experiments were performed in the presence of Ca^{2+} Green-5N probe. Fluorescence (*F*) is expressed in A.U. and represents extramitochondrial Ca^{2+}. Ca^{2+} was added every 120 s by increments of 20 nmol/injection. Displayed graphs are representative of three independent experiments. The values in *E* are presented as means ± S.D. nmol Ca^{2+}/mg protein of three independent experiments (Kruskal–Wallis; $H = 7.624$, $p = 0.0357$), followed by Dunn's post-test. *a*, $p < 0.05$ *versus* basal.

ryanodine (Fig. 4*E*). These results are consistent with the previous observation from Fig. 3 and confirm our estimation that caffeine/ryanodine-induced reticular Ca^{2+} release represented ~50% of the Ca^{2+} amount necessary to open mPTP.

One of the most studied cardioprotective strategy within the last decade has relied on either the pharmacological inhibition or the genetic ablation of cyclophilin D, which, under Ca^{2+} stimulation, is known to enhance mPTP opening. Assuming that the reticular Ca^{2+} content is stable, we wondered whether CypD inhibition/suppression would also shift the amount of extracellular Ca^{2+} required to open mPTP in our experimental model. We first determined the amount of Ca^{2+} necessary to open mPTP after CypD inhibition/suppression and then loaded cardiomyocytes with 50% of this amount prior to inducing reticular Ca^{2+} release with 10 nM ryanodine (Fig. 5*A*). When CsA was added to the preparation, an additional 63.3 ± 14.5 nmol Ca^{2+}/mg protein were necessary to induce mPTP opening after ryanodine stimulation (Fig. 5*B*, *blue plot*). If rotenone, a complex I inhibitor, was added to CsA, the amount of Ca^{2+} required to open mPTP was raised to 133.7 ± 27.0 nmol Ca^{2+}/mg protein (Fig. 5*C*, *blue plot*). Similar results were observed in CypD-KO adult cardiomyocytes (Fig. 5*D*, *blue plot*), in which the addition of 177.7 ± 15.3 nmol Ca^{2+}/mg protein was necessary to induce mPTP opening. Compared with the control condition tested in Fig. 4*C* (where no additional Ca^{2+} was needed to open mPTP after stimulation of reticular Ca^{2+} release), the inhibition/suppression of CypD in our model significantly increase mitochondria capacity to uptake Ca^{2+} (Fig. 5*E*).

Effect of reticular Ca^{2+} mobilization on mPTP opening after AR

Altogether, our results emphasized the major contribution of both internal Ca^{2+} stores and extracellular Ca^{2+} to mitochondrial/cellular fate in normoxic condition. We next assessed whether AR would modify this dynamic.

Cardiomyocytes were subjected to 30 min of anoxia followed by a 15-min reoxygenation period (in the presence or absence of 1 mM extracellular Ca^{2+}) prior to CRC measurement. As a control (sham groups), cardiomyocytes were incubated for 45 min in normoxic condition also in the presence or absence of 1 mM extracellular Ca^{2+} (Fig. 6*A*).

AR protocol was determined based on our previous work and on a recent study from Panel *et al.* (22), to guarantee a sufficient amount of viable cardiomyocytes after AR and to achieve a valuable measurement of mPTP opening. The amount of Ca^{2+} needed to open mPTP was determined (as in Fig. 3) either using only Ca^{2+} pulses addition (sham groups and AR1 groups) or

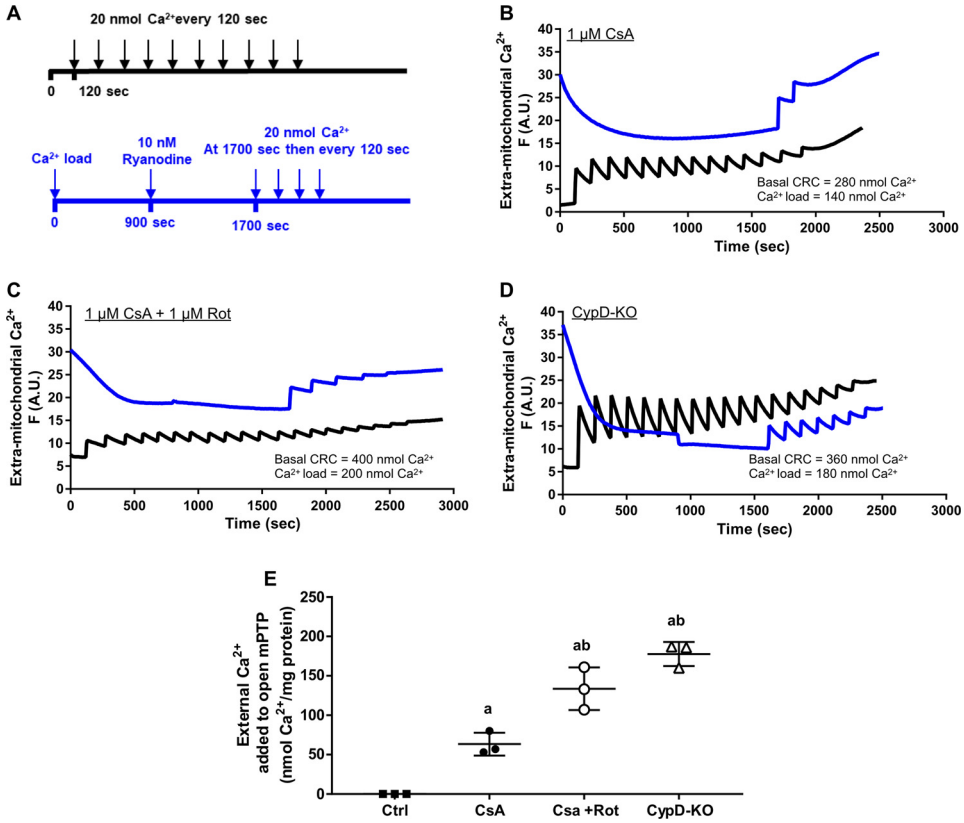


Figure 5. Effects of pharmacological and genetic protection on mPTP opening. *A*, Ca^{2+} amount necessary to induce mPTP opening was determined without reticular Ca^{2+} stimulation (*black*) or after stimulation with 10 nM ryanodine (*blue*). *B–D*, measurements were performed in the presence of 1 μM CsA (*B*) or 1 μM CsA + 1 μM rotenone (*C*) in WT adult cardiomyocytes or in CypD-KO adult cardiomyocytes (*D*). CRC was measured after the addition of 50% of the amount of Ca^{2+} necessary to open the mPTP. This amount was calculated based on basal CRC (*black plots*) with each CypD inhibition or suppression. At 900 s, ryanodine receptors were stimulated with 10 nM ryanodine. 900 s later, fluorescence level did not increase, indicating no mPTP opening. Further pulses of 20 nmol of Ca^{2+} were added to induce mPTP opening (*B–D*, *blue plots*). *E*, amount of Ca^{2+} added to open mPTP after ryanodine stimulation: in control (without CypD inhibition/suppression (*i.e.* Fig. 4*C*) or when CypD is inhibited/suppressed. The data are presented as means ± S.D. nmol Ca^{2+}/mg protein of three independent experiments: one-way ANOVA ($F(3, 8) = 62.41$; $p < 0.0001$), followed by Tukey post-test. *a*, $p < 0.05$ *versus* control; *b*, $p < 0.005$ *versus* CsA. All experiments were performed in the presence of Ca^{2+} Green-5N probe. Fluorescence (*F*) is expressed in A.U. and represents extramitochondrial Ca^{2+}. Ca^{2+} was added every 120 s by increments of 20 nmol/injection. Displayed graphs are representative of three independent experiments.

using 5 mM caffeine (to induce reticular Ca^{2+} release) followed by Ca^{2+} pulse addition (AR2 groups) (Fig. 6*A*).

As expected, the amount of Ca^{2+} added to open mPTP decreased after AR protocol. AR1 with or without 1 mM Ca^{2+} compared with respective sham (Fig. 6*B*): 108 ± 19 *versus* 210 ± 35 nmol Ca^{2+}/mg protein ($p < 0.0001$; with 1 mM extracellular Ca^{2+}) and 193 ± 22 *versus* 290 ± 40 nmol Ca^{2+}/mg protein ($p < 0.0001$; without 1 mM extracellular Ca^{2+}). In AR2, our results showed that even after AR protocol, either with or without 1 mM extracellular Ca^{2+}, reticular Ca^{2+} release after caffeine stimulation was not sufficient to open mPTP. Additional Ca^{2+} pulses were added to trigger mPTP opening.

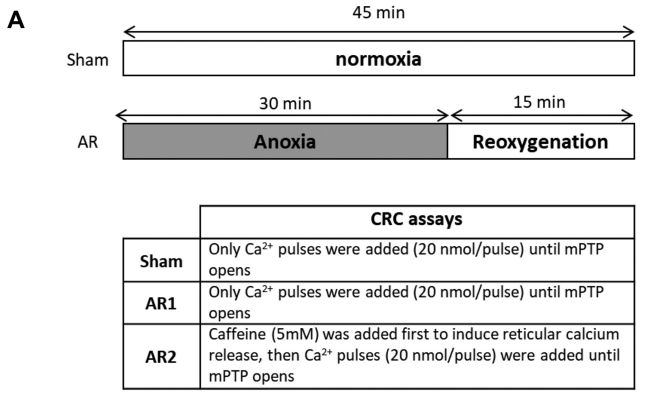
Moreover, in the presence of 1 mM extracellular Ca^{2+}, CRC values significantly decreased when compared with the respective condition in the absence of 1 mM extracellular Ca^{2+} (sham, AR1, and AR2 with 1 mM extracellular Ca^{2+} *versus* sham, AR1, and AR2 without 1 mM extracellular Ca^{2+}, respectively; $p <$ 0.0001). Strikingly, in none of these conditions were intracellular Ca^{2+} stores sufficient to trigger mPTP opening, even though the presence of extracellular Ca^{2+} during AR facilitates mPTP opening. In all these conditions, the addition of exogenous Ca^{2+} pulses was needed to induce mPTP opening. Interestingly, other biophysical features can be evaluated using our measurement method, which is described below.

The mobilized internal Ca^{2+} stores—These stores can be estimated by measuring the difference between CRC value in sham without extracellular Ca^{2+} and the CRC value in AR2 without extracellular Ca^{2+} (Table 1). The mobilized internal Ca^{2+} stores in our model would be ~187 ± 38 nmol Ca^{2+}/mg protein.

The remaining internal Ca^{2+} stores after AR—The measured CRC values without 1 mM extracellular Ca^{2+} were significantly different in AR1 compared with AR2 ($p < 0.005$). Therefore, the remaining internal Ca^{2+} stores after AR can be estimated by subtracting CRC value in AR1 from CRC value in AR2 without 1 mM extracellular Ca^{2+} and were equivalent to 80 ± 39 nmol Ca^{2+}/mg protein (Table 1).

The proportion of internal Ca^{2+} stores leak during AR—Knowing both the total internal Ca^{2+} amount (187 ± 38 nmol Ca^{2+}/mg protein) and the remaining internal Ca^{2+} after AR (80 ± 39 nmol Ca^{2+}/mg protein), we can estimate the internal Ca^{2+} that leaked during AR to 57 ± 23% (Table 1).

The extracellular Ca^{2+} amount that entered the cell during AR—The difference between the CRC values of AR2 in the absence and the presence of 1 mM extracellular Ca^{2+} represents



	CRC assays
Sham	Only Ca^{2+} pulses were added (20 nmol/pulse) until mPTP opens
AR1	Only Ca^{2+} pulses were added (20 nmol/pulse) until mPTP opens
AR2	Caffeine (5mM) was added first to induce reticular calcium release, then Ca^{2+} pulses (20 nmol/pulse) were added until mPTP opens

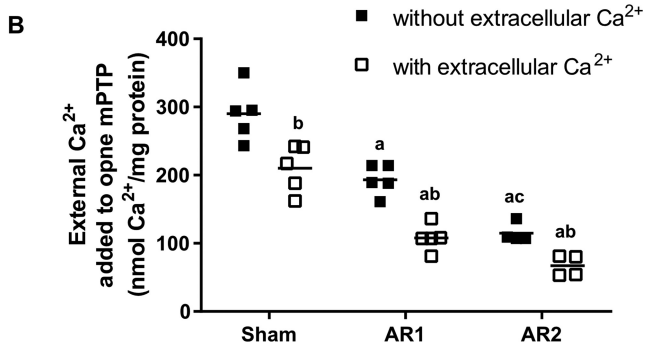


Figure 6. CRC value variations after AR in the absence or presence of extracellular Ca^{2+}. *A*, design of the AR protocol and CRC measurement methods are presented. Adult cardiomyocytes were stored for 45 min in normoxic conditions (sham groups) or subjected to 30 min of anoxia followed by 15 min of reoxygenation (AR groups). Both sham and AR were performed in the presence or in the absence of 1 mM extracellular Ca^{2+}. Two methods were used to measure CRC: in sham groups and AR1 groups, only Ca^{2+} pulses were added (20 nmol/pulse) until mPTP opens. In AR2 groups, caffeine (5 mM) was added first to induce reticular Ca^{2+} release, and then Ca^{2+} pulses were added (20 nmol/pulse) until mPTP opens. *B*, values of Ca^{2+} amount added to open mPTP are presented as means ± S.D. nmol Ca^{2+}/mg protein of four or five independent experiments. Two-way ANOVA was conducted on the influence of the presence of 1 mM extracellular Ca^{2+} on mPTP opening in the different above protocols. Both AR ($F(2, 22) = 81.27$; $p < 0.0001$) and 1 mM Ca^{2+} addition ($F(1, 22) = 48.2$; $p < 0.0001$) had a significant effect on CRC. Tukey post-test showed significant differences: *a*, $p < 0.0001$ *versus* respective sham; *b*, $p < 0.0001$ with extracellular Ca^{2+} *versus* without extracellular Ca^{2+}; *c*, $p < 0.005$ AR1 *versus* AR2. The interaction was, however, not significant ($F(2, 22) = 1.228$; $p = 0.3121$). *Ctrl*, control.

an estimation of the amount of extracellular Ca^{2+} that entered the cells during AR and was equal to 48 ± 14 nmol Ca^{2+}/mg protein (Table 1). The 48 ± 14 nmol Ca^{2+}/mg protein represented approximately half of the internal Ca^{2+} stores that leaked to the mitochondria (187 − 80 = 107 nmol Ca^{2+}/mg protein). Overall, we can conclude that in our experimental conditions, two-thirds of the Ca^{2+} loaded to the mitochondria during AR may come from internal Ca^{2+} stores (mainly from the reticulum), whereas approximately one-third seems to be of extracellular origin.

Discussion

Mitochondria are involved in multiple cell processes such as the control of cytoplasmic Ca^{2+} level because of their Ca^{2+} uptake capacity. This participation is particularly important when the cellular environment is subjected to major stress such as ischemia–reperfusion during which the absence of oxygen induces the drop of ATP synthesis and consequently the inhibition of SERCA activity contributing thus to several physiological mishandling including a massive intracellular Ca^{2+} overload (23). In such a situation, mPTP transient activity could act as a safety valve, which enables Ca^{2+} leak with an increase rate of efflux. Ultimately, this mechanism triggers mitochondrial swelling, which has been associated with cell death. This finely tuned regulation, balanced by Ca^{2+} and mediated by CypD, has been extensively studied (24–26). However, in most of the studies, no distinctions were made regarding the origin of Ca^{2+} (internal Ca^{2+} stores and/or extracellular Ca^{2+}) inducing the opening of mPTP, more likely because there was no reliable method to investigate it.

The proposed method in this study is derived from classical CRC assay and proposes a simple mean to quantitatively estimate the respective contribution of internal Ca^{2+} stores and extracellular Ca^{2+} toward mPTP opening. CRC can be achieved by the addition of exogenous Ca^{2+} pulses to isolated mitochondria (27) or permeabilized cells (28). However, this classical CRC method does not allow the study of reticular Ca^{2+} input. In parallel to CRC experiment, most of the standard protocols use Ca^{2+} ionophores and Ca^{2+} addition to induce mPTP opening. These Ca^{2+} ionophores can induce artifacts in cellular models including in isolated adult cardiomyocytes (22) and can also induce a release of internal Ca^{2+} stores in a nonspecific manner, as compared with caffeine or ryanodine treatment, which specifically induces reticular Ca^{2+} release.

Interestingly, our presented method relies on the analysis of mitochondria–reticulum interaction *in situ*. We used freshly isolated adult murine cardiomyocytes as a cellular model. Cardiomyocytes plasma membranes were permeabilized with 40 μM digitonin. This chemical agent and the used concentration are both respectful of the intracellular membrane structure (29–31). The intracellular microdomains and organelle interactions are thus preserved in this model, allowing the study of mitochondria–reticulum interaction within an integrated physiological-like cellular environment (31, 32). This was also shown by confocal microscopy images showing no impact of 40 μM digitonin on the mitochondrial network in our model (Fig. 1*E*). We also confirmed the reliability of our cellular model and our experimental conditions, as well as the absence of Ca^{2+} Green-5N fluorescence bleaching throughout the duration of our experiments.

In our permeabilized model, the low ATP level ensures a natural inhibition of SERCA activity and therefore excludes reticular Ca^{2+} uptake (33). In addition, with this method, one can activate the reticular Ca^{2+} release via ryanodine receptors with specific agonists such as caffeine or ryanodine (34, 35) to estimate its involvement in mPTP opening. In isolated adult cardiomyocytes, we estimated that reticular Ca^{2+} represents ~50% of the Ca^{2+} amount necessary to open mPTP in basal conditions. This proportion may represent an indicator of physiological Ca^{2+} steady state between the mitochondria and the reticulum. A second biophysical parameter that can be deduced with this method is the proportion of Ca^{2+} content that leaks from the internal stores during AR: ~57% during a 15-min reoxygenation. Finally, the third biophysical parameter that can be estimated is the proportion of extracellular Ca^{2+} entering the cells during AR, which represents ~50% of the

Table 1
Estimation of the mobilized Ca^{2+} cross-talk during AR
The values represent means ± S.D. of four or five independent experiments.

Mobilized internal Ca^{2+} stores	Remaining internal Ca^{2+} stores	Proportion of internal stores Ca^{2+} leak during AR	External Ca^{2+} amount that entered the cell during AR
(Sham without Ca^{2+}) − (AR2 without Ca^{2+})	(AR1 without Ca^{2+}) − (AR2 without Ca^{2+})	100 − [(2) × 100/(1)]	(AR2 without Ca^{2+}) − (AR2 without Ca^{2+})
187 ± 38 nmol/mg protein	80 ± 39 nmol/mg protein	57 ± 23%	48 ± 14 nmol/mg protein
(1)	(2)	—	—

internal Ca^{2+} leaking during AR protocol. Considering these two last parameters, we can suggest that ~15–20% of the total Ca^{2+} content inducing mPTP opening comes from the extracellular Ca^{2+} pulses added during the CRC experiments measured after AR protocol. To our knowledge, this is the first demonstration that internal Ca^{2+} leaks during AR require additional factors like external Ca^{2+} to open mPTP.

Another advantage offered by this method is the quantification estimate of the relationship between extracellular Ca^{2+} content and steady-state mitochondrial Ca^{2+} content. It has always been assumed that Ca^{2+} homeostasis translates extracellular Ca^{2+} changes to shifts in Ca^{2+} concentration in the cytosol and organelles. Although the quantification of this interdependence could be realized in live cells with fluorescent probes, the calibration of these probes in mitochondria is much harder than in the cytosol. With our method, we found that the CRC value in cardiomyocytes incubated for 45 min (sham groups) in the absence of extracellular Ca^{2+} was increased by 80 nmol Ca^{2+}/mg protein compared with an incubation in the presence of 1 mM extracellular Ca^{2+}. This emphasizes the fact that extracellular Ca^{2+} contribute to the mitochondrial Ca^{2+} homeostasis. Future experiments will be performed as a dose-effect response to fully quantify and understand this interdependence.

One limitation of our method could be that a proportion of the Ca^{2+} released by caffeine leaks in the medium instead of entering the mitochondria. However, this would mean that upon caffeine stimulation, the fluorescence of Ca^{2+} Green-5N probe should rise. The dissociation constant of Ca^{2+} Green-5N probe is ~14 μM. In our experimental conditions, the caffeine-mediated Ca^{2+} released is ~70 μM (187 nmol Ca^{2+}/mg protein with 750 μg of protein in 2 ml of CRC medium). It is thus very unlikely that the probe could not detect a reticular Ca^{2+} leak, if there is any. Along the same line, it should be noted that the *insets* in Fig. 3 (*B* and *C*) showed no significant Ca^{2+} variation in the cytosol, suggesting that it was rapidly taken up by the mitochondria, whereas a fluorescence increase was observed in the mitochondria as shown by a Rhod2-AM probe, which is sensitive to free Ca^{2+} variation (insets Fig. 3*F*). This was also confirmed by the absence of reticular Ca^{2+} uptake in the presence of the mitochondrial uncoupler FCCP (Fig. 3*D*) and mitochondrial Ca^{2+} uptake inhibitor, RU360 (Fig. 3*E*). These results highlight the efficiency of mitochondria–reticulum connection to channel Ca^{2+} between both organelles in isolated adult cardiomyocytes.

Another limitation is that in our experimental model, CICR is very limited because of cellular permeabilization and very low ATP amount. However, in the situation of ischemia–reperfusion injury, the absence of ATP production makes CICR unlikely to occur, suggesting the absence of CICR contribution to mPTP opening during ischemia–reperfusion (36) similarly to our experimental model.

Finally, pH restoration during reperfusion, one of the factors that could participate in enhancing mPTP opening (11, 37), is probably underestimated in our experimental model because of the use of buffered medium in AR protocol. Further experiments are needed to assess any synergetic activation of mPTP with this factor.

In this work, we showed that the mitochondrial Ca^{2+} level required to open mPTP exceeded the reticular Ca^{2+} content in both sham and AR experimental conditions either when the protocol was performed in the presence or in the absence of 1 mM extracellular Ca^{2+}. In all these conditions, additional Ca^{2+} pulses were needed to induce mPTP opening. This work emphasizes the prior knowledge that both extracellular Ca^{2+} and internal Ca^{2+} stores are important triggers of mPTP opening, by discerning, estimating, and contextualizing their respective contribution. Our results highlight the involvement of extracellular Ca^{2+} to mitochondrial Ca^{2+} homeostasis but also suggest that the mPTP opening process requires additional mechanisms such as protein and membrane oxidation by reactive oxygen species (38) or pH restoration (10, 11).

This original and simple approach could be applied to a large panel of cell types and cellular models to detect *in situ* reticulum–mitochondria Ca^{2+} transfer dysfunction and would open new perspectives for the study and screening of pharmacological molecules for mitochondrial and reticular targets. This method could thus bring new and valuable insights into the physiopathological investigation of numerous diseases.

Experimental procedures

Animals

We obtained 8–12-week-old male C57BL/6J mice from Charles River Laboratories (L'arbresle, France). CypD-KO mice under C57Bl/6/SV129 background were issued from Korsmeyer's laboratory (Dana Farber Cancer Institute, Boston, MA). CypD-KO male mice (8–12 weeks old, 20–30 g) were obtained by homozygous intercross in our laboratory.

The present study was in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication 85-23, revised 1996), and all experiments were approved by the University of Lyon Ethics Committee (UCBL1 approval BH2007-07).

Cardiomyocytes isolation

Adult mouse cardiomyocytes were freshly isolated using two different enzymatic digestions as previously described (39, 40). Briefly, hearts from cervically dislocated male mice were

quickly removed and retrogradely perfused with Krebs–Henseleit buffer. Ventricular cardiomyocytes were isolated using enzymatic digestion with 0.167 mg·ml^{-1} Liberase (Roche) and 0.14 mg·ml^{-1} trypsin 2.5% (Invitrogen), or with 2.4 mg·ml^{-1} collagenase type II (Gibco). Cellular protein concentration was measured using the Bradford method. Isolated cardiomyocytes viability was between 85 and 95%. The cells were used within 5 h after isolation.

Mitochondria isolation

Isolated mitochondria were used as part of the validation of the study model. After being removed, the hearts were quickly placed in an ice-cold isolation buffer (70 mM sucrose, 210 mM mannitol, and 10 mM EGTA, 50 mM Tris-HCl, pH 7.4). Myocardial tissue was finely minced and then homogenized in the same buffer. Mitochondria were isolated in accordance with our previous studies (39, 41). Briefly, the homogenate was centrifuged at 1300 × *g* for 3 min, and the supernatant was centrifuged at 10,000 × *g* for 10 min. The mitochondrial pellet was then suspended in a cold buffer containing: 70 mM sucrose and 210 mM mannitol in 50 mM Tris-HCl, pH 7.4, and centrifuged at 10,000 × *g* for 10 min. Mitochondrial protein concentration was measured using the Bradford method. Mitochondria were used within 3 h after isolation.

Ca^{2+} retention capacity assay

This study was mainly based on the measurement of CRC, an *in vitro* surrogate for the susceptibility of mPTP opening following a Ca^{2+} overload. Briefly, the sample was placed in the CRC medium (150 mM sucrose, 50 mM KCl, 2 mM KH_2PO_4, 20 mM Tris-HCl, and 5 mM succinate-Tris) and under continuous stirring in the spectrofluorometer. After 120 s of stabilization time, 20 nmol of $CaCl_2$ (pulses) were added every 120 s. Modification of extramitochondrial Ca^{2+} concentration was continuously recorded in the presence of a Ca^{2+} fluorescent probe. After sufficient Ca^{2+} loading, an increase in fluorescence intensity represents an elevation of extramitochondrial Ca^{2+} concentration, indicating a massive release of Ca^{2+} by mitochondria caused by mPTP opening. The amount of Ca^{2+} necessary to trigger a massive Ca^{2+} release, expressed as the CRC value, was used as an indicator of mPTP susceptibility to Ca^{2+} overload.

CRC assay can be performed on isolated mitochondria or permeabilized cells and uses only exogenous Ca^{2+} addition to achieve mitochondrial Ca^{2+} overload. The purpose of the present study was to evaluate mitochondria–reticulum Ca^{2+} cross-talk.

Consequently, (*a*) CRC was specifically performed on a model of permeabilized cardiomyocytes to benefit from the entire intracellular environment. (*b*) Intracellular Ca^{2+} store release was triggered using caffeine or ryanodine addition. Another specification of the permeabilized cell context is the very low ATP amount. ATP is diluted in the assay medium and is not sufficient to induce SERCA activity (33), which guarantees no refilling of the sarco/endoplasmic stores and prevent CICR. (*c*) CRC assay was then performed as previously described. Ca^{2+} fluorescence was measured using a spectrofluorometer (F-2500, Hitachi, High-tech) with two different Ca^{2+} probes: Ca^{2+} Green-5N and Rhod2-AM (Invitrogen). (*d*) Ca^{2+} Green-5N probe (excitation, 506 nm; emission, 530 nm) detects extramitochondrial calcium. Cardiomyocytes (750 μg of protein) were added in 2 ml of CRC medium. This medium was supplemented with 0.4 μM Ca^{2+} Green-5N and 40 μM digitonin for cardiomyocytes permeabilization (42). (*e*) Rhod2-AM probe (excitation, 551 nm; emission, 582 nm) detects free intramitochondrial Ca^{2+} and attests that Ca^{2+} was actually taken up by the mitochondria. Cardiomyocytes (750 μg of protein) were incubated with 5 μM of Rhod2-AM for 30 min at room temperature and then washed for 30 min with a buffer containing 50 mM Tris-HCl, 70 mM saccharose, and 210 mM of mannitol, pH 7.4, to remove remaining Rhod2-AM, as well as Ca^{2+} traces.

Confocal microscopy

Cardiomyocytes (750 μg of protein) were incubated with 5 μM of Rhod2-AM and 0.2 μM MitoTracker Green under the same conditions described above. Images were taken on a confocal microscope Nikon A1r using an oil-immersion 40× objective (N.A. 1.3). MitoTracker Green and Rhod2-AM were excited with 488 and 560-nm wavelength laser lines, respectively. The emitted light was filtered by 525 ± 25 and 595 ± 25 bandpass filters, respectively. 1024 × 1024 pixels images were acquired with an average of four scanning lines.

Anoxia–reoxygenation protocol

The effect of depleting reticulum Ca^{2+} stores on mPTP opening (as described above) was evaluated after AR in the presence of Ca^{2+} Green-5N. Cardiomyocytes (750 μg of protein) were subjected to 30 min of hypoxia followed by 15 min of reoxygenation in the presence or absence of 1 mM extracellular Ca^{2+} (nonpermeabilized cardiomyocytes). Anoxia was achieved using CRC medium (with or without 1 mM Ca^{2+}) degassed for 5 min with nitrogen and supplemented with 0.5 mM dithionite. At the end of anoxia, the cells were washed and centrifuged at 20 × *g* for 3 min and resuspended with 2 ml of fresh oxygenated CRC medium. Sham groups (cardiomyocytes placed for 45 min in normoxic conditions) were subjected to centrifugation/resuspension and used as controls for AR groups. At the end of reoxygenation time, CRC was measured following two different procedures: either using only Ca^{2+} pulses addition (Sham groups and AR1 groups) or after the addition of 5 mM caffeine first (to release reticular Ca^{2+}) followed by the addition of Ca^{2+} pulses to complete the measurement (AR2 groups).

Data processing and presentation

The animals were randomly distributed between groups. The data were analyzed using GraphPad Prism 6 (GraphPad software, San Diego, CA). Displayed graphs are representative of three independent experiments and presented as fluorescence arbitrary unit (A.U.) as a function of time in seconds.

Histograms and scatter plots are represented as means ± S.D. nmol Ca^{2+}/mg protein of three to five distinct experiments. Interactions and comparisons between groups were made using one-way ANOVA or two-way ANOVA followed by Tukey multicomparison post-test. Kruskal–Wallis followed by

Dunn's multicomparison post-test were used as nonparametric tests in absence of Gaussian normality. The values for $p < 0.05$ were considered significant.

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